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Morphologic and molecular data help adopting the insect-pathogenic nephridiophagids (Nephridiophagidae) among the early diverging fungal lineages, close to the Chytridiomycota

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Abstract

Nephridiophagids are poorly known unicellular eukaryotes, previously of uncertain systematic position, that parasitize the Malpighian tubules of insects. Their life cycle includes merogony with multinucleate plasmodia and sporogony leading to small, uninucleate spores. We examined the phylogenetic affiliations of three species of *Nephridiophaga*, including one new species, *Nephridiophaga maderae,* from the Madeira cockroach (*Leucophaea maderae*). In addition to the specific host, the new species differs from those already known by the size of the spores and by the number of spores within the sporogenic plasmodium. The inferred phylogenetic analyses strongly support a placement of the nephridiophagids in the fungal kingdom near its root and with a close, but unresolved, relationship to the chytids (Chytridiomycota). We found evidence for the nephridiophagidean speciation as being strongly coupled to host speciation.

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Key words
Cryptomycota, entomoparasitic, entomopathogenic, Fungi, Haplosporidia, Microsporidia, Molecular phylogeny, protozoa, Rozellomycota, small subunit ribosomal DNA (SSU, 18S), spore morphology

Introduction

Arthropods may be infected by a range of unicellular pathogens of disparate taxonomic affiliations (Lange and Lord 2012). The majority of entomopathogenic spore-forming protists belong to the supertaxa Opisthokonta (e.g. Microsporidia) and SAR (Alveolata with the Apicomplexa; Rhizaria with the Haplosporidia and Paramyxea; Adl et al. 2012). Nephridiophagids (Nephridiophagidae) are unicellular, spore-forming parasites previously of uncertain systematic position. They infect the Malpighian tubules of insects and are mainly found in the lumen of these tubules (e.g., Woolever 1966, Radek and Herth 1999). The life cycle of nephridiophagids includes a merogony phase with vegetative multinucleate plasmodia that divide into oligonucleate and uninucleate cells. Sporogonial plasmodia form internal, 5–10 µm long, oval, flattened spores, generally with one nucleus. Residual nuclei of the mother cell remain in the cytoplasm between the developing spores.

The systematic position of the nephridiophagids has been discussed intensively. Morphologically, this lineage could not be assigned unambiguously to any of the known major taxa of spore-forming protists. Some authors place them with the haplosporidians (Ivanić 1937, Woolever 1966, Purrini and Weiser 1990) while others disagreed with this grouping (Toguebaye et al. 1986, Purrini and Rhode 1988, Lange 1993). With the aid of a light microscope, the nephridiophagid stages resemble microsporidians (Microsporidia), and by tradition some nephridiophagids have been given names in microsporidian genera (e.g., *Nosema periplanetae* and *Pleistophora periplanetae*; Lutz and Splendore 1903, Perrin 1906). A preliminary molecular analysis placed them within the Fungi, close to 'zygomycota' (Wylezich et al. 2004, White et al. 2006). Since then, the Microsporidia have been placed near the root of the fungal kingdom (Capella-Gutiérrez et al. 2012, Xiang et al. 2014) as have the Cryptomycota (Lazarus and James 2015). The genus *Nephridiophaga* was introduced by Ivanić (1937) for *N. apis*, which infects honey bees. Insects, which represent the metazoan group with the highest species richness, appear to be remunerative to screen for novel fungal taxa which were hidden in habitats insulated from the free environment (Hawksworth 2001).

The Fungi comprise upwards of 6 million extant species, of which some 135,400 have been described formally (Blackwell et al. 2011, Hibbett et al. 2011, Taylor et al. 2014; www.speciesfungorum.org as of May 2017). Although all true fungi are heterotrophs, they occupy a very wide range of niches and nutritional modes. About 1% of the described species – 750-1,000 species from about 100 genera – are pathogens of insects. These entomopathogens are distributed over most fungal phyla, and their hosts are spread among 20 orders of insects (Araújo and Hughes 2016). All insect developmental stages from egg to adult may be subject to infection. Molecular data have

increased our understanding of insect-fungal relationships considerably. A wide range of associations and infection types has been discovered, ranging from parasitic through commensal and even beneficial (Suh et al. 2005, Vega et al. 2012, Douglas 2015). High-throughput sequencing is rapidly gaining in popularity as a means of studying fungus-insect interactions, and published studies have uncovered surprising diversity even within single insect individuals (e.g., Dhami et al. 2013). This is in line with the results from other environmental fungal sequencing efforts, where tens to hundreds of previously unknown (or at least not sequenced) species are usually found in each new study undertaken (Nilsson et al. 2016). It is thus not speculative to assume that a significant number of insect pathogenic fungi await discovery and formal description.

Many early diverging fungi are associated with insects, however, this region of the fungal tree of life suffers from poor taxon sampling and phylogenetic resolution. The last few years have seen the description of numerous new species and lineages of early diverging fungi, even at the phylum level (e.g., James et al. 2006, Corsaro et al. 2014, Karpov et al. 2014a, b, Bauer et al. 2015). The nephridiophagids belong in this part of the fungal kingdom (Wylezich et al. 2004), but they have yet to be addressed using phylogenetic methods in the context of a rich taxon sampling of closely related taxa. The present study uses a molecular phylogenetic approach to examine the phylogenetic relationships of the nephridiophagids. We included three species of *Nephridiophaga* from cockroaches, viz. *N. blattellae*, *N. blaberi,* and a new species from the Madeira cockroach (*Leucophaea maderae*). Increasing the number of analyzed species we aim to clarify the relationships among the deep lineages of the Fungi. Our molecular, morphological, and ultrastructural results show that the nephridiophagids may represent a distinct clade at the root of the Fungi.

Materials and Methods

Animal material

Specimens of the Death´s Head Cockroach *Blaberus craniifer*, the German Cockroach *Blattella germanica,* and the Madeira Cockroach *Leucophaea* (*Rhyparobia*) *maderae* were retrieved from the Federal Environment Agency (UBA; https://www.umweltbundesamt.de/en) in Berlin, Germany. Cockroaches of different ages and sex were dissected, and their Malpighian tubules were removed and processed for further examination through light and electron microscopy as well as molecular analysis.

Light microscopy

For fresh preparations, parts of the tubules were ground with fine forceps in a drop of 0.6% NaCl solution. The infected tubules were then smeared on a microscopic slide, air dried, and fixed in methanol for 5 min prior to staining with Giemsa solution (Ac-

custain, Sigma; 1:10 in tap water for 45 min). Dried smears were mounted in Entellan (Merck). Extracted bundles of Malpighian tubules were embedded in paraffin (Paraplast) for histological examination. Fixation was carried out in Bouin's fluid, modified after Dubosq-Brasil (Böck 1989). Sections of 7 µm were stained with hematoxylin-eosin (Böck 1989) and embedded in Malinol (Chroma). The chitinous spore walls of native spores were fluorescently labeled with 0.01% Calcofluor White M2R in a 50 mM phosphate buffer of pH 7.2 for 15 min. Photos were taken with a Zeiss Axiophot equipped with an Inteq digital camera and the software EasyMeasure 1.4.

Scanning electron microscopy

Cover glasses were coated with 0.01% poly-L-lysine to promote attachment of spores. Malpighian tubules were ground in a drop a fixative (1% OsO_4, 2.5% glutardialdehyde, 0.1 M cacodylate buffer, pH 7.2) on the cover glasses and fixed for 1 h. After dehydration in a graded series of ethanol, the prepared cover glasses were critical point dried in a Baltec CPD 030 and sputtered with gold in a Baltec SCD 040. Images were taken with a Quanta 200 scanning electron microscope from FEI Company.

Transmission electron microscopy

Stages of *N. blattellae* were fixed (glutaraldehyde, reduced osmium) and embedded according to Radek and Herth (1999).

DNA extraction

For molecular analysis of the nuclear small subunit (SSU, 18S) rRNA encoding rDNA sequences of microscopically identified *Nephridiophaga* species, dissected Malpighian tubules of *Blattella germanica, Blaberus craniifer,* and *Leucophaea maderae* were transferred into 1.5 ml PCR-clean reaction tubes (Eppendorf, Hamburg, Germany) with 50 µl of distilled water and stored at -20°C pending further analysis. Alternatively, the tubules were put into 50 µl of lysis buffer. (0.5% sodium dodecyl sulfate, 200 mM TRIS-HCl pH 8.0). For DNA extraction, specimens were centrifuged at 13,200 *g* for 5 min using the Eppendorf benchtop centrifuge 5415R with a F45-24-11 rotor. The supernatant was removed, and total DNA was extracted from the obtained pellets using the DNeasy Plant Mini Kit from Qiagen (Hilden, Germany). Briefly, each pellet was thoroughly resuspended in 400 µl of warm buffer AP1 and 4 µl RNase A (100 mg/ml). Samples were incubated at 65°C for 10 min and 20 min at room temperature. Next, 130 µl of AP2 buffer was added and samples were incubated for 5 min on ice. Lysate was transferred into the QIA shredder column and the column was centrifuged

for 2 min at 13,200 *g*. The flow-through was gently mixed with 1.5 volume AP3/E buffer, transferred to a DNeasy spin column, and centrifuged for 1 min at 6000 *g*. The column was placed into a new 2 ml collecting tube and washed with 500 µl AW buffer. The column was centrifuged for 1 min at 6,000 *g*, after which the flow-through was removed and the column was washed again with 500 µl AW buffer. Centrifugation was performed at 13,200 *g* for 2 min. Finally the column was placed into a 1.5 ml PCR-clean reaction tube and DNA was eluted with 50 µl AE buffer. After 5 min incubation at room temperature, the column was centrifuged at 6,000 *g* for 2 min. The extracted DNA was stored at -20°C pending further analysis.

For amplification of the SSU sequences of *N. blattellae*, *N. blaberi,* and *N. maderae*, the eukaryotic universal primers published by Medlin et al. (1988) without polylinker were used that span the complete 18S (Table 1). Additionally, we designed a bridging *Nephridiophaga*-specific primer (Nephbla3 rv) based on the public SSU sequence of *N. blattellae* (NCBI GenBank accession no. AY603958) using PrimerBLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Since *Nephridiophaga* DNA was extracted from ground cockroaches, primer specificity was essential, so that the primers do not match the host. The tiny amount of fungal DNA compared to host DNA could lead to preferential amplification of cockroach DNA unless specific primers were used. The primers used targeted nucleotide position 1-21 (Euc Uni 18S fw), nucleotide position 872-891 (Nephbla3 rv and Nephbla3 fw) and nucleotide position 1787-1810 (Euc Uni 18S rv) of the complete 18S ribosomal RNA gene of *N. blattellae* (NCBI GenBank accession no. AY603958), resulting in sequences of 891 nt (Euc Uni 18S fw and Nephbla3 rv) and 939 nt (Nephbla3 fw and Euc Uni 18S rv) (Table 1). All primer sets were synthesized by Eurofins MWG Operon (http://www.eurofinsgenomics.eu/).

PCR amplification of SSU rDNA

PCR amplification of the 18S rRNA gene was performed using HotStarTaq *Plus* DNA polymerase kit (Qiagen) and 10 mM dNTP mix (Peqlab, Erlangen, Germany) according to the manufacturers' protocols. PCR reactions were performed with an initial DNA denaturation step at 95°C for 5 minutes followed by 35 cycles of 94°C for 1 min, 59°C for 1 min for each primer set (Table 1), 72°C for 1 min, and a final elongation step at 72°C for 10 min. Amplification products were separated on a 1% agarose gel, stained with ethidium bromide, and visualized under UV light.

Table 1. Primer sets used to amplify the SSU rDNA of *Nephridiophaga blattellae*, *N. blaberi,* and *N. maderae.*

Source	Primer	Sequence 5′-3′	Product size
Medlin et al. 1988	Euc Uni 18S fw	AACCTGGTTGATCCTGCCAGT	891 nt
this study	Nephbla3 rv	AATACTGACGCCCCCAACTG	
this study	Nephbla3 fw	CAGTTGGGGGCGTCAGTATT	939 nt
Medlin et al. 1988	Euc Uni 18S rv	TGATCCTTCTGCAGGTTCACCTAC	

Sequencing of amplified the SSU rDNA

The PCR amplicons were purified using the QIAquick PCR Purification Kit from Qiagen. Briefly, 20 µl of each PCR-product was resuspended in 100 µl PB-buffer and transferred to a QIAquick DNA column, centrifuged at 16,100 *g* for 30 s and the flow-through was aspirated. The column was washed with 750 µl PE-buffer and centrifuged at 16,100 *g* for 30 s. The flow-through was aspirated and the column was centrifuged at 16,100 *g* for 30 s to remove any residual ethanol. The column was placed into a 1.5 ml PCR-clean reaction tube (Eppendorf), and 50 µl of warm EB-buffer was added onto the membrane. To elute the PCR amplicons, the columns were centrifuged at 8,000 *g* for 2 min. The purified PCR products were sent to Eurofins-Genomics (http://www.eurofinsgenomics.eu/) for sequencing. The short sequences (Table 1) were edited and processed using the VectorNTI software from Invitrogen™ Life Technologies (Darmstadt, Germany). Two new SSU rDNA sequences were generated, one each for *N. blaberi* (1,697 bases) and *N. maderae* (1,784 bases). These were deposited in GenBank (Benson et al. 2017) under accession numbers KU900289-KU900290. For further phylogenetic analysis we also used the *N. blattellae* SSU rDNA sequence AY603958 (1,807 bases) from GenBank.

Phylogenetic inference

The generated sequences were aligned against the SILVA SSU reference database (v119) using SINA (Pruesse et al. 2012), which accounts for secondary structures of the ribosomal RNA. We added the zygomycete sequences from White et al. (2006) to the reference database to increase the coverage of fungal lineages at the root of the fungal kingdom. For the general placement of *Nephridiophaga* into the eukaryotic tree of life we took the multiple sequence alignment of all 62k reference database entries and removed all overly short sequences as well as sequences with anomalies (Ashelford et al. 2005), thus reducing the dataset to 40k entries with 22,404 analyzed characters. A maximum likelihood tree with the multithread version of FastTree (version 2.1, Price et al. 2010) was inferred, specifying 10k resamplings using a GTR model. *Nephridiophaga* was recovered within the Holomycota (syn.: Nucletmycea; Suppl. material 1, Fig. S1), and we thus compiled a representative dataset of 196 taxa from all Holomycota and close neighbors. The final alignment was adjusted manually in AliView 1.17 (Larsson 2014). We only allowed full length sequences ranging from SILVA SSU position 1132 to 43048 for all subsequent phylogenetic inferences, including all variable regions, resulting in a total of 2,773 analyzed characters, of which 1,057 were invariable. Phylogenetic inference was done using Bayesian inference in MrBayes v. 3.2.6 (Ronquist et al. 2012) and maximum likelihood in FastTree with 10,000 bootstrap replicates. The Bayesian inference of phylogeny was based on 20 million generations under the GTR model and INVGAMMA substitution rates as suggested by MrModelTest 2.3 (Nylander et al. 2004). Chain mixing and convergence were satisfactory (the latter approaching an average split frequency of 0.008). Sequence similarities were

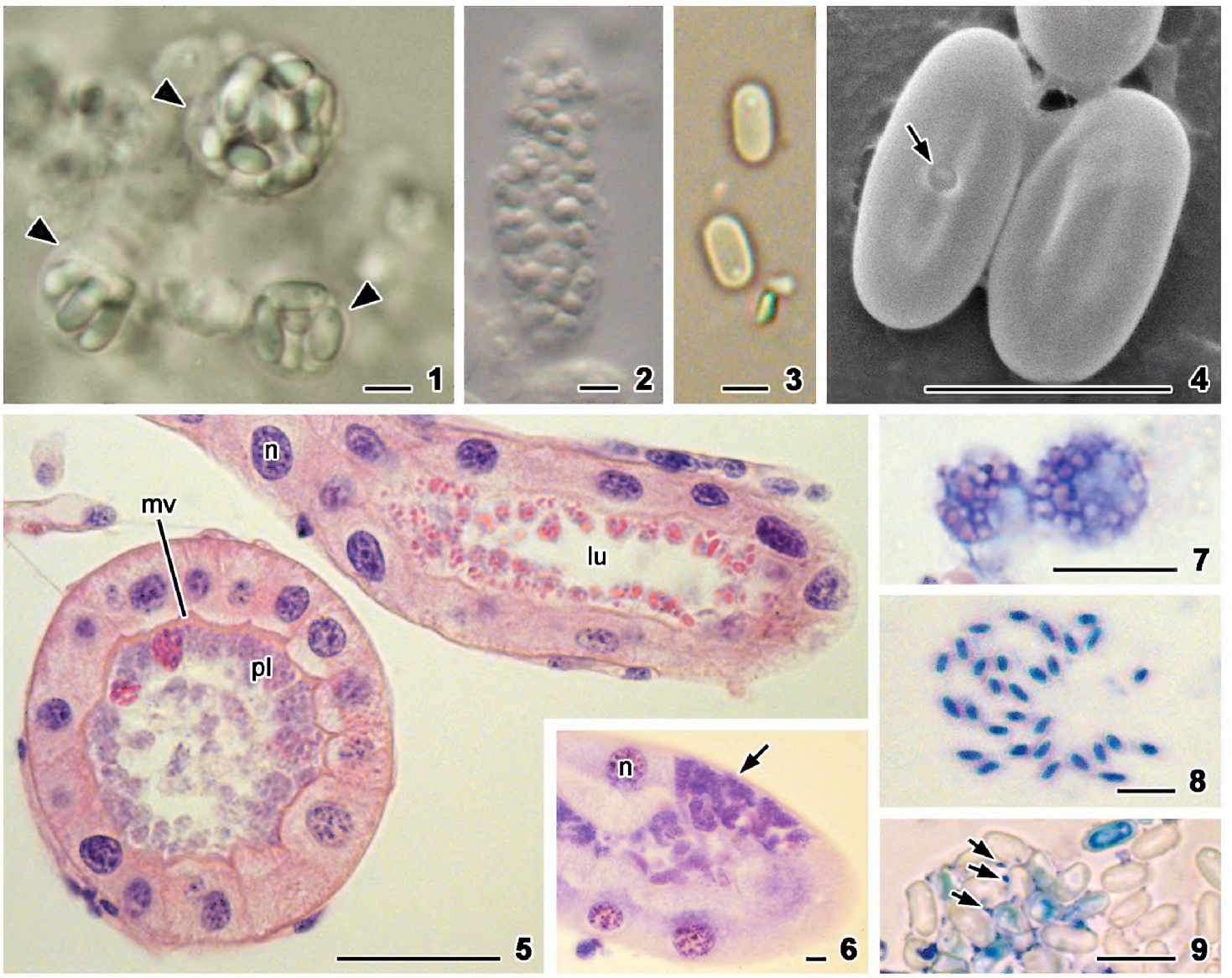
calculated based on Jukes and Cantor (1969) distances. The multiple sequence alignment and the phylogenetic trees were deposited in TreeBASE at https://treebase.org/ (study no. S19000).

Results

Characterization of the new species Nephridiophaga maderae

While species of *Nephridiophaga* from *Blattella germanica* (Blattellidae, Blattellinae) and *Blaberus craniifer* (Blaberidae, Blaberinae) were already known, there is no formal description of a nephridiophagid from *Leucophaea maderae* (Blaberidae, Oxyhaloinae). In nine out of ten dissected Madeira cockroaches, the Malpighian tubules were infected by a spore-forming nephridiophagid. The degree of infection was generally low (6–10 sec of microscopy necessary before finding first stages). Two animals were infected more heavily (1–5 sec of microscopy). None of the infected cockroaches showed obvious symptoms of illness. In fresh smears, spore-containing plasmodia (Fig. 1), vegetative multinucleate plasmodia (Fig. 2), and single spores were seen, which jointly form the typical stages of species from the genus *Nephridiophaga.* The number of spores in a sporogenic plasmodium varied between 6 and 26, with a mean number of 15 (n = 34). As long as the plasma membrane of the plasmodium is intact (Fig. 1, arrows), the spores are kept together in groups. Single spores have a flattened oval form, measuring 6.3–7.9 (7.2) x 3.1–4.7 (3.7) µm in fresh preparations (n = 50) and 4.8–7.5 (6.4) x 2.4–4.5 (3.3) µm in Giemsa-stained smears (n = 50). Scanning electron micrographs reveal a centrally localized, plugged spore opening on the upper side (Fig. 4, left spore). The lower side has no opening but may be slightly folded (Fig. 4, right spore). The rim of the spore is thickened. In hematoxylin-eosin stained paraffin sections, the localization of the parasites in the lumen of the Malpighian tubules can be seen clearly (Fig. 5). Many cells attach to the microvilli border of the epithelial cells while others are free in the lumen. Only very rarely, intracellular vegetative plasmodia are found in the epithelial cells of the Malpighian tubules (Fig. 6). Giemsa staining of smears also reveals the different stages, viz. multinucleated vegetative plasmodia (Fig. 7), young spores whose interior can be stained (Fig. 8), and mature spores into which the stain cannot penetrate. Typical for nephridiophagids are the residual vegetative nuclei of the mother cell in the cytoplasm between the spores (Fig. 9).

Characteristic ultrastructural features of the genus *Nephridiophaga* are demonstrated using the example of *N. blattellae* (Figs 10–13). Vegetative plasmodia have a variable cell form and contain one to several nuclei, numerous mitochondria, and an endoplasmic reticulum (Fig. 10). The mitochondria are of the tubular to sac-like type rather than of the cristae type. Sporogenic plasmodia internally form flattened-oval, thick-walled spores with one nucleus; residual vegetative nuclei remain in the cytoplasm of the mother cell (Fig. 11). The spores contain typical eukaryotic cell structures such as a nucleus, mitochondria, and an endoplasmic reticulum but no obvious extra elements (Fig. 12). A layer of small vesicles attaching to the lining of the developing

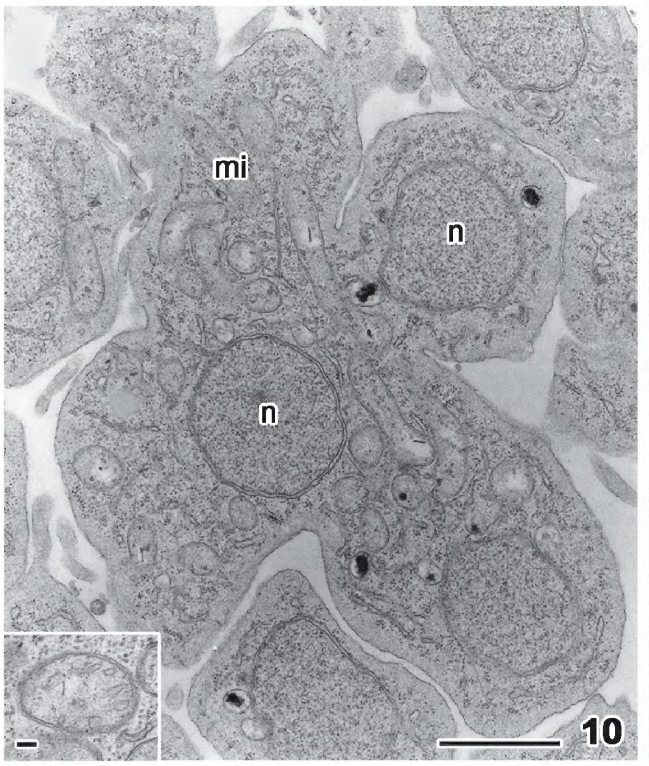


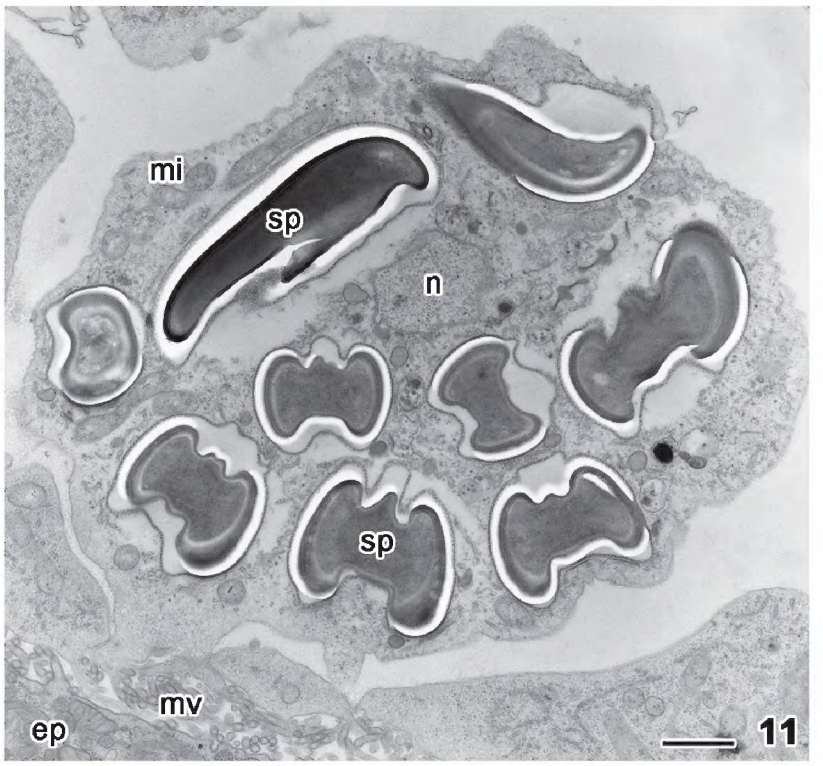
Figures 1–9. *Nephridiophaga maderae*, **1, 2, 5–9** bright field **3** phase contrast **4** scanning electron microscopy. **1** Three sporogonial plasmodia with different numbers of included spores. Arrows point to plasma membrane. **2** Merogonial plasmodium with numerous nuclei. **3** Mature spores. **4** The upper surface of the spore possesses a central spore opening (arrow, left spore) while the lower surface of the spore lacks an opening (right spore). **5, 6** Paraffin sections stained with hematoxylin-eosin. Generally, the plasmodia (pl) are found in the lumen of the Malpighian tubule but are often attached to the microvilli (mv) (**5**). Rarely, aggregates of vegetative plasmodia (arrow) occur in the epithelial cells of the Malpighian tubules (**6**). n = nuclei of epithelial cells. **7–9** Smears of macerated tubules stained with Giemsa depicting vegetative plasmodia (**7**), stained young spores (**8**), and unstained mature spores with residual nuclei (arrows) of the mother sporoplasm. Scale bars: 5 μm (**1–4**), 50 μm (**5**), 10 μm (**6–9**).

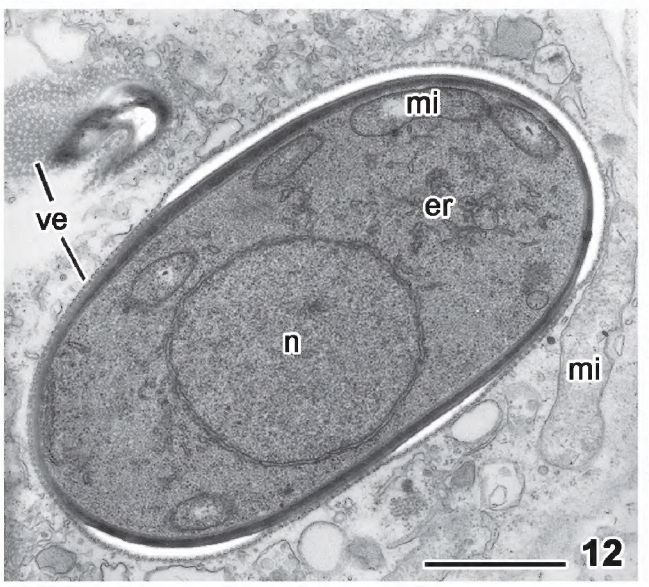
sporoblasts is probably involved in the formation of the spore wall (Fig. 12). The only structure apparently aiding in hatching of the sporoplasm is a central spore opening through which the sporoplasm can escape (Fig. 13). Calcofluor staining reveals the presence of chitin in the spore wall (Fig. 14).

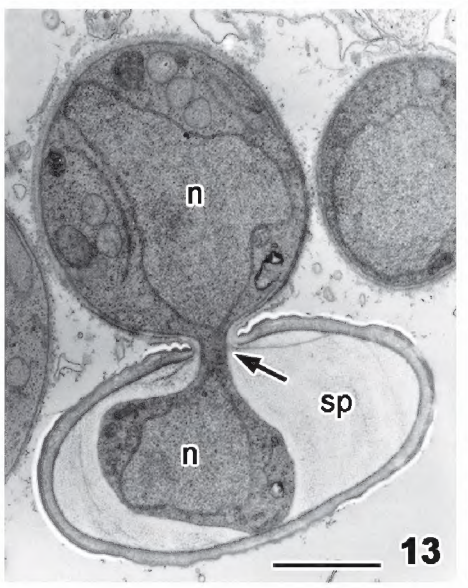
Phylogenetic position of Nephridiophaga

Since we wanted to clarify the phylogenetic relationship of *Nephridiophaga* with respect to other spore-forming pathogens, we included members of the former Zygomycota as well as the Haplosporidia and Microsporidia (Suppl. material 1, Fig. S1). The genus









Figures 10–14. *Nephridiophaga blattellae*, **10–13** transmission electron microscopy, **14** Calcofluor white staining. **10** Meront with several nuclei (n) and mitochondria (mi) in the lumen of Malpighian tubule. Inset: Mitochondrium with tubular to sac-like cristae. **11** Sporogenic plasmodium containing mature spores (sp), mitochondria (mi), and vegetative nuclei (n) in the cytoplasm. The plasmodium is anchored to the microvilli (mv) of epithelial cells (ep) of the tubule. **12** Young spore within the cytoplasm of a sporogenic plasmodium, surrounded by a layer of vesicles. The spore cytoplasm contains one nucleus (n), mitochondria (mi), and endoplasmic reticulum (er). **13** An infectious sporoplasm hatches through the central spore opening, leaving behind the spore wall of the emptying spore (sp). The nucleus (n) is squeezed through the tiny spore opening. **14** Calcofluor white stains the spore wall indicating the presence of chitin (bluish color). Scale bars: 1 μm (**10–13**), inset 0.1 μm (**10**), 5 μm (**14**).

Nephridiophaga is clearly positioned within the Fungi but does not cluster together with any of the long branches of Microsporidia (Cryptomycota), Haplosporidia (SAR group), or *Dimargaris* (Dimargaritales, Kickxellomycotina, 'zygomycota'). We further selected a representative set of entries from the Holomycota phyla and its sister clades in order to find the most probable position of *Nephridiophaga* in the backbone tree. Again we recovered strong support for the *Nephridiophaga* within the Fungi (Fig. 15).

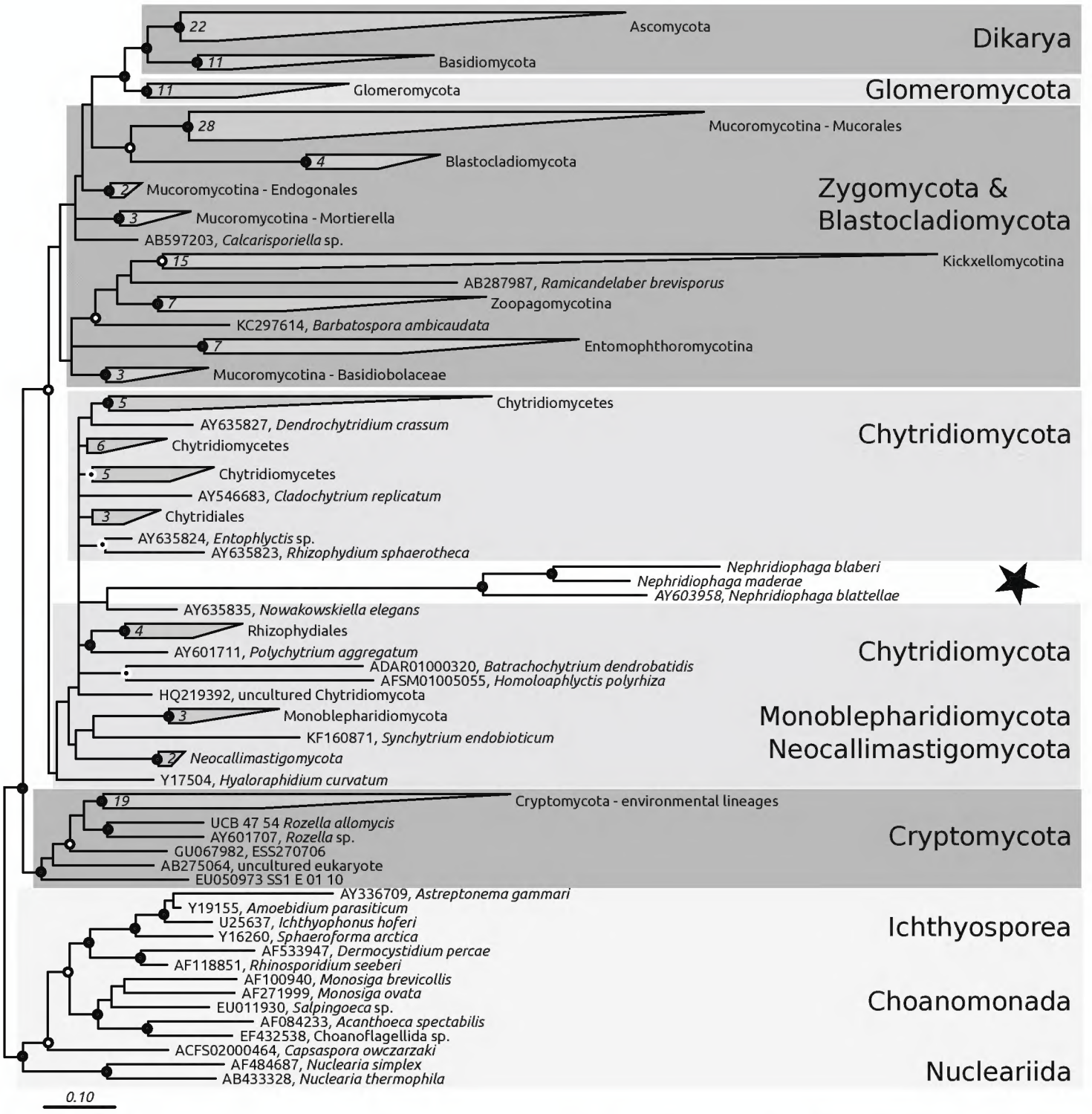


Figure 15. Bayesian phylogenetic tree including major lineages of the Holomycota (Liu et al. 2009; syn.: *Nucletmycea*, Brown et al. 2009), i.e. Fungi, Cryptomycota, and the basal Nucleariida, together with Holomycota sister clades Choanomonada, Ichthyosporea, and Filasterea (Holozoa; Lang et al. 2002). *Nephridiophaga* species (star) form a clade together with the flagellate fungi, here indicated as Chytridiomycota *s.l.* The scale indicates expected changes per site. Branch support is given as Bayesian posterior probabilities above 0.95 (black circles) and maximum likelihood resampling values above 90% (white circles). Filled black circles mark support from both methods.

The clade could not be assigned to the Cryptomycota but instead formed a clade with the Chytridiomycota *sensu lato*. The fully supported branch leading to *Nephridiophaga* points to an independent lineage near the root of the fungal kingdom. In addition, the strong branch support obtained for data from each of the three samples of *Nephridiophaga* from different cockroach species supports the notion that the isolates indeed

represent three distinct species. The new species *Nephridiophaga maderae* differed from described taxa by approximately 12-14% at the intrageneric level and by more than 20% at the inter-phylum level in its small subunit ribosomal DNA sequence (as referred to pairwise sequences similarity). The *Nephridiophaga* clade is not known from environmental sequences.

Taxonomy

***Nephridiophaga maderae* Radek, Owerfeldt, Gisder & Wurzbacher sp. nov.**
MycoBank: MB552000

Diagnosis. Flattened, oval to elongate, uninucleate spores measuring 6.3–7.9 (7.2) x 3.1–4.7 (3.7) µm in fresh preparations and 4.8–7.5 (6.4) x 2.4–4.5 (3.3) µm in Giemsa-stained smears. 6–26 (15) spores per sporogenic plasmodium. Vegetative and sporogenic life cycles stages in lumen of Malpighian tubules. Vegetative plasmodia are rarely intracellular in epithelial cells of Malpighian tubules.

Holotype. Two slides were deposited in the Upper Austrian Museum in Linz, Austria (Giemsa stained smear with slide number 2014/58 and hemalaun-eosin stained paraffin sections with slide number 2014/59).

Distribution / host locality. Culture at the Federal Environment Agency (UBA), Berlin, Germany. Naturally occurring in tropical regions world-wide.

Ecology: Infection of the host by oral ingestion of spores. Life cycle stages develop in the Malpighian tubules. Spores released via the feces.

Etymology and host. Named after its host, the Madeira cockroach, *Leucophaea maderae*.

Discussion

The phylogenetic position of *Nephridiophaga* has been a longstanding enigma in the systematics community. As a result of the re-appraisal of fungal phylogeny during the Deep Hypha project (Blackwell et al. 2006), *Nephridiophaga blattellae* was reported to cluster among the fungi and not among other eukaryotes as previously described using the SSU sequence generated by Wylezich et al. (2004). *Nephridiophaga blattellae* appeared to have some statistically supported phylogenetic relationship with the Kickxellales-Dimargaritales-Zoopagales clade among the zygomycetes (White et al. 2006). Here we report the generation of SSU sequence data from another species which was previously described, *N. blaberi*, and from the new species of *Nephridiophaga* in order to re-evaluate the phylogenetic position for the nephridiophagids. We were able to provide robust phylogenetic support (100%) for the position of the nephridiophagids near the root of the fungal kingdom.

The identification of Nephridiophaga maderae *as novel species*

All nephridiophagids found so far in cockroaches belong to the genus *Nephridiophaga*: *N. archimandrita* (Radek et al. 2011), *N. blaberi* (Fabel et al. 2000), *N. blattellae* (Crawley 1905, Woolever 1966, Radek and Herth 1999), *N. lucihormetica* (Radek et al. 2011), *N. periplanetae* (Lutz and Splendore 1903, Lange 1993), and *N. tangae* (Purrini et al. 1988). Characteristics of these species were compiled and tabulated by Radek et al. (2011) and the species studied so far seem to be host specific. Furthermore, they differ slightly in the size of spores and the number of spores within the sporogenic plasmodium. The localization of the life stages is mostly in the lumen of the Malpighian tubules, but in some species intracellular vegetative plasmodia have also been found. Due to these characters we believe that an as-yet unknown species of *Nephridiophaga* occurs in *Leucophaea maderae*. Woolever (1966) already mentioned the occurrence of a nephridiophagid in this host but did not provide any details. Our sequence data strongly support the existence of this un-named species and the general occurrence of different species of nephridiophagids in different hosts, indicating that speciation in *Nephridiophaga* is strongly linked to host speciation. The new species differs from *N. blattellae* by 12% and from *N. blaberi* by 14% in their nuclear small subunit ribosomal DNA which is a sufficient phylogenetic distance, according to Marshall and Berbee (2011), to justify a new species. *Nephridiophaga maderae* sp. nov. joins four other species of *Nephridiophaga* in having been recovered from cockroaches (Woolever 1966, Fabel et al. 2000, Radek et al. 2011), hinting at the unique and diverse life forms that can be found by investigating taxa inhabiting divergent host species.

Phylogenetic position of the genus Nephridiophaga

The results confirmed the finding of Wylezich et al. (2004) that *Nephridiophaga* belongs to the fungal kingdom rather than being related to non-fungal eukaryotes. It was, however, not possible to resolve the branching order of the non-Dikarya fungi in a robust way. We found a polytomy where the nephridiophagids were embedded within the flagellate fungi, the Chytridiomycota sensu lato (Voigt 2012). There is strong phylogenetic and ultrastructural support not to assign the nephridiophagids to any of the newly erected/redefined phyla Blastocladiomycota, Chytridiomycota *sensu stricto*, Monoblepharidomycota, and Neocallimastigomycota, all of which stem from the former phylum Chytridiomycota *s.l.* (see Voigt (2012) for an overview).

It is interesting that sequences from species of *Nephridiophaga* have never been recovered in studies based on environmental sequencing, although primer mismatches can be hypothesized to be the culprit (cf. Tedersoo et al. 2015). Alternatively, *Nephridiophaga* sequences may be rare enough to be below the detection limit in bulk environmental samples. So far, we have only recovered sequences from the cockroach clade (Blattodea), which is an early-diverging lineage. Thus, species of *Nephridiophaga* from other arthropods will be extremely helpful to retrace the evolutionary history

of this cryptic and enigmatic group of fungi. During the past, the placement of one novel fungal group (Archaeorhizomycetes) discovered using DNA-based methods shifted when more characters from additional rDNA and protein-coding regions were added to the analysis (Rosling et al. 2011). Multiple markers such as the nuclear large subunit (LSU, 28S) ribosomal DNA (rDNA) in addition to the nuclear small subunit (SSU, 18S) ribosomal DNA sequences and protein coding genes will be very helpful for future phylogenetic efforts involving the *Nephridiophaga* clade. Indeed, the LSU has been proposed as a good genetic marker for non-Dikarya fungi (e.g., Letcher et al. 2006). Obtaining additional genes for *Nephridiophaga* is, however, very laborious and resource intensive, given the endobiotic nature of these minute fungi. Herein, we decided to opt for SSU rDNA sequences with the aim to expose the uniqueness of *Nephridiophaga.* Increased research interest in this genus and related lineages will hopefully bring about the developments needed in primer design to support the generation of additional genetic marker data, and even full genomes, to fully resolve the precise phylogenetic position of *Nephridiophaga* within the kingdom Fungi. We are in the process of generating additional ribosomal (ITS and LSU) and nuclear gene sequences (Elongation factor alpha) for *Nephridiophaga.*

While about 98% of the described fungi belong to the Dikarya, comprising the two phyla Ascomycota and Basidiomycota, the relationships among the remaining lineages of fungi are less well resolved (Carr and Baldauf 2011, Bauer et al. 2015). Our molecular analyses provide strong support for *Nephridiophaga* as a distinct lineage closely related to the Chytridiomycota *s.l. Nephridiophaga* probably originates from flagellate fungi but has secondarily lost its flagella. Morphological and ultrastructural characters that support the inclusion of *Nephridiophaga* in the Fungi include a heterotrophic lifestyle, propagation by spores, an intranuclear position of the spindle during nuclear division, the presence of chitin in the spore wall, and chitosome-like vesicles on the surface of the maturing spore (Radek et al. 2002).

Significant rDNA sequence divergence above 20%, distinctive morphology, and unique life cycle traits support the delimitation of *Nephridiophaga* from other fungus-like organisms – the ARM clade (Aphelida, Cryptomycota, and Microsporidia; cf. Karpov et al. 2014a, Corsaro et al. 2016) – found near the root of the kingdom Fungi. The ARM clade is presently not included in the Fungi. Nephridiophagids are different from Microsporidia by not possessing a polar tube, polaroplast, and posterior vacuole – structures that are involved in the hatching process of Microsporidia. Endoparasitic trophonts of the Aphelida and Cryptomycota (syn.: Rozellomycota) are able to phagocytose whereas nephridiophagids do not engulf particulate food (Powell 1984, Karpov et al. 2013). The morphology and the life cycle of *Nephridiophaga* deviate from the Chytridiomycota *s.l.,* which possesses typical flagellate stages (zoospores) and centrioles. These structures are missing in nephridiophagids. The only microtubules detected in nephridiophagids so far are intranuclear spindle microtubules formed during nuclear division (Radek and Herth 1999). Thus, the morphology of the kinetosome-associated structures useful in the determination of families and genera of zoosporic fungi cannot be used for classification here (Powell and Letcher 2014). In

general, the Blastocladiomycota, Chytridiomycota *s.str.*, Neocallimastigomycota, and Monoblepharidomycota – the Chytridiomycota *s.l.* – develop posterior flagellate zoospores when free-living but lose the flagella when the life cycle is endoparasitic in all stages (James et al. 2006, Voigt 2012, Powell and Letcher 2014). The nephridiophagids appear to have lost the ability to produce flagella, much like the endoparasitic Microsporidia. This may be due to the completely endobiotic life style, which renders active motility less of a useful trait.

In contrast to many other organisms at the root of the Fungi, the habitat of nephridiophagids is quite restricted – they represent one of the comparatively few groups of fungal endoparasites of arthropods known so far. Further morphological differences are the lack of mycelia (a thallus) and microbody-lipid complexes (MLCs). The MLCs, assemblages of lipid globules, endoplasmic reticulum, mitochondria, and microbodies in Chytridiomycota *s.l.* are suggested to be involved in the conversion of energy from lipids (Powell 1976, Powell and Letcher 2014). Lipid globules to date have not been identified in nephridiophagids, and the presence of microbodies is similarly unclear. In *N. blattellae*, opaque vesicles with homogenous content have been observed (Radek and Herth 1999), but catalase activity could not been shown (unpublished, RR). Compared to agile zoospores, the various life stages of nephridiophagids probably do not need much, if any, energy for motility. Nephridiophagids possess mitochondria with tubular to sac-like inner membrane structures, which stands in strong contrast to mitochondria with cristae in the other fungi. On the whole, there are no specific morphological traits in nephridiophagids that can be used to support a close relationship with chytridiomycete lineages. Molecular analyses presently seem to be the only clue to resolve their phylogenetic relationships – and the molecular results support the nephridiophagids as a distinct lineage among the early diverging fungi.

Conclusion

The molecular, morphological, and ultrastructural evidence brought forward in this study point to the fact that the nephridiophagids form a distinct clade of fungi whose precise taxonomic affiliation cannot be settled at the present time. But we refrain from assigning a rank to this clade in the context of insufficient sampling and clade stability.

Fortunately, we have come far in the generation of molecular data from additional genes and genetic markers, such that we hope to be able to resolve the phylogenetic position of the nephridiophagids in the not too distant future. Studies closing in on the very root of the Fungi have the potential to cast light not only on those particular lineages, but also on the evolution of all extant fungal groups and their nutritional modes and biotic interactions. The increasingly ambitious sampling efforts undertaken by the mycological and molecular ecology communities leave no doubt that the next few years will witness substantial scientific progress in understanding and delimiting the root of the kingdom Fungi. Targeting the cockroach habitat will be worthwhile to consider for future environmental sequencing studies.

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Supplementary material 1

Figure S1

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Data type: molecular data

Explanation note: SSU phylogenetic tree of all eukaryotic lineages (40 k sequences) including spore-forming protist taxa and fungal groups. The *Nephridiophaga* species (purple) cluster closely to Cryptomycota and several long branches of Chytridiomycota and 'zygomycota' sequences at the base of the fungal tree. Holomycota sequences are yellow whereas Haplosporidia and Microsporidia are highlighted in red. The scale indicates expected changes per site. Branch support is given as maximum likelihood resampling values.

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